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New vouchered and taxonomically verified records of cave-dwelling populations of catfishes of the genus *Rhamdia* (Siluriformes, Heptapteridae) from Costa Rica

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Abstract

Dedicated ichthyological surveys in two karstic caves in Costa Rica resulted in the discovery of hypogean populations from three epigean species of catfishes of the genus *Rhamdia*. The taxonomic identity of these populations was initially determined based on morphological traits and subsequently corroborated with comparative DNA sequence data in a phylogenetic framework. Individuals from all hypogean populations documented herein exhibit only partial troglomorphism, characterized by only moderate (vs. complete) integumentary depigmentation without extreme eye reduction/loss. A similar pattern of incomplete troglomorphism at the individual level has been observed in other cave-dwelling species/populations of Middle American *Rhamdia*, and tentatively attributed to gene flow with and/or incipient

speciation from epigean lineages. Since most hypogean forms of *Rhamdia* derive from/are part of a larger clade of primarily *R. laticauda*, our discovery of cave-dwelling populations assignable to *R. nicaraguensis* and *R. guatemalensis* is noteworthy, particularly in the case of the former, which represents the first taxonomically verified record of a cave-dwelling population of this epigean species. Among our findings is the remarkable discovery of hypogean populations from two different species of *Rhamdia* (*R. laticauda* and *R. nicaraguensis*) inhabiting the same cave (Gabinarraca). This finding is particularly significant because it represents the first time that cave-dwelling populations from different species of *Rhamdia* are reported to be living in syntopy. Continued discovery of cave-dwelling populations during targeted ichthyological surveys reinforces the notion that our understanding of the diversity of hypogean *Rhamdia* is incomplete and that sustained exploration and taxonomically sound documentation work are paramount to advancing knowledge about the diversity and evolution of these group of Neotropical catfishes.

Keywords
Cavefishes, hypogean, stygobionts, stygofauna, troglomorphism

Introduction

With 27 currently valid species distributed throughout most of the tropical Americas, catfishes of the genus *Rhamdia* Bleeker 1858 are a group of moderately diverse Neotropical freshwater fishes noteworthy, among other things, because of their tendency to colonize subterranean waters and maintain resident hypogean populations (Hubbs 1938; Silfvergrip 1996; Perdices et al. 2002; Bichuette and Trajano 2005; Hernández et al. 2015; Arroyave and De La Cruz Fernández 2021a, 2021b; Buenavad-González et al. 2023; Fricke et al. 2024). In fact, several hypogean populations of *Rhamdia* have been described as distinct species, recognized as different from each other and from any of their epigean counterparts (Miller 1984; Wilkens 1993; Weber and Wilkens 1998; Weber et al. 2003; DoNascimiento et al. 2004; Bichuette and Trajano 2005), although recent research has called into question the taxonomic validity of some of these cave-adapted species (Arroyave and De La Cruz Fernández 2021b).

Despite its widespread distribution, the bulk of the subterranean diversity of the genus *Rhamdia* has been primarily described from karstic regions in Mexico. Mexican cave *Rhamdia* includes four of the six currently valid troglobitic species in the genus plus at least 10 cave-dwelling populations taxonomically assigned, for the most part, to the epigean species *R. laticauda* (Kner 1858) (Arroyave and De La Cruz Fernández 2021a, 2021b, Buenavad-González et al. 2023). Recent efforts dedicated to investigating the taxonomic diversity and evolution of Middle American species of *Rhamdia* have shown that the existence of cave-dwelling populations is more geographically widespread than previously thought and that exploration and dedicated ichthyological surveys of underground karstic caves and their associated aquatic environments is likely to result in the discovery of novel subterranean populations, further supporting the idea that cave colonization in the group is widespread (Arroyave and De La Cruz Fernández 2021a, Buenavad-González et al. 2023). Discovery and documentation of novel hypogean populations not only entails the un-

covering of new biodiversity, but also brings forth additional comparative material for future research on the systematics and biogeography of the genus *Rhamdia*. This material (specimens and tissue samples) is key to shed light on the evolution of cave colonization and troglomorphism, intriguing topics that can be studied from both morphological and genetic perspectives.

Whereas recent studies have uncovered and documented the existence of previously unknown cave-dwelling populations of *Rhamdia* in Mexico (Arroyave and De La Cruz Fernández 2021a, Buenavad-González et al. 2023), similar efforts appear to be lacking for the remainder of the distribution of the genus. Costa Rica, a small yet megadiverse country in southern Central America (Avalos 2018), is a part of a natural bridge between North and South America and home to three epigean species of *Rhamdia*: *R. guatemalensis* (Günther 1864), *R. laticauda*, and *R. nicaraguensis* (Günther 1864) (Fricke et al. 2024). Despite harboring several species of *Rhamdia* and having over 250 documented caves (Ulloa et al. 2011), accounts of cave-dwelling populations of *Rhamdia* from Costa Rica are rare, mostly reported by speleologists and mainly supported by anecdotal evidence (Strinati et al. 1987; Debeljak 1988; Juberthie and Strinati 1994; Sandí 2012; Deleva et al. 2023), sometimes including photographs, but never taxonomically verified voucher specimens catalogued in ichthyological collections. Lack of vouchered evidence when documenting hypogean fish fauna—and all biodiversity for that matter—is less than ideal because accurate taxonomic identifications often rely on the direct, close, and careful examination of morphological traits (Rocha et al. 2014; Ceríaco et al. 2016; Nachman et al. 2023). In the case of populations potentially representing undescribed species, the need of voucher specimens is even more pressing. Furthermore, vouchers, when tissued prior to formalin fixation, allow for further taxonomic verification using molecular data, which can also be used in a comparative framework to investigate an assortment of evolutionary questions that require genetic markers.

Historical accounts of hypogean *Rhamdia* from Costa Rica include reported sightings of cave-dwelling forms of *R. guatemalensis* (five sites), *R. nicaraguensis* (one site), and *Rhamdia* sp. (four sites) (Deleva et al. 2023). None of these, however, constitute taxonomically authoritative/verified records (i.e., supported by voucher specimens—not just observations—from which morphology- and/or DNA-based taxonomic identifications have been conducted), thus rendering them potentially unreliable (Rocha et al. 2014; Ceríaco et al. 2016; Nachman et al. 2023). These reports, nonetheless, offer an important baseline to plan and conduct targeted ichthyological surveys aimed at determining the existence and taxonomic identity of any residing cavefish populations.

In a first effort towards properly and accurately documenting the taxonomic diversity of cave *Rhamdia* in Costa Rica, we conducted dedicated ichthyological surveys in two karstic caves with anecdotal reports on the presence of these catfishes: Corredores and Gabinarraca (or Cavernas de Venado) (Fig. 1). This study presents our findings, focusing on the taxonomic nature and phylogenetic placement of these populations, as inferred from morphological and molecular comparative data.

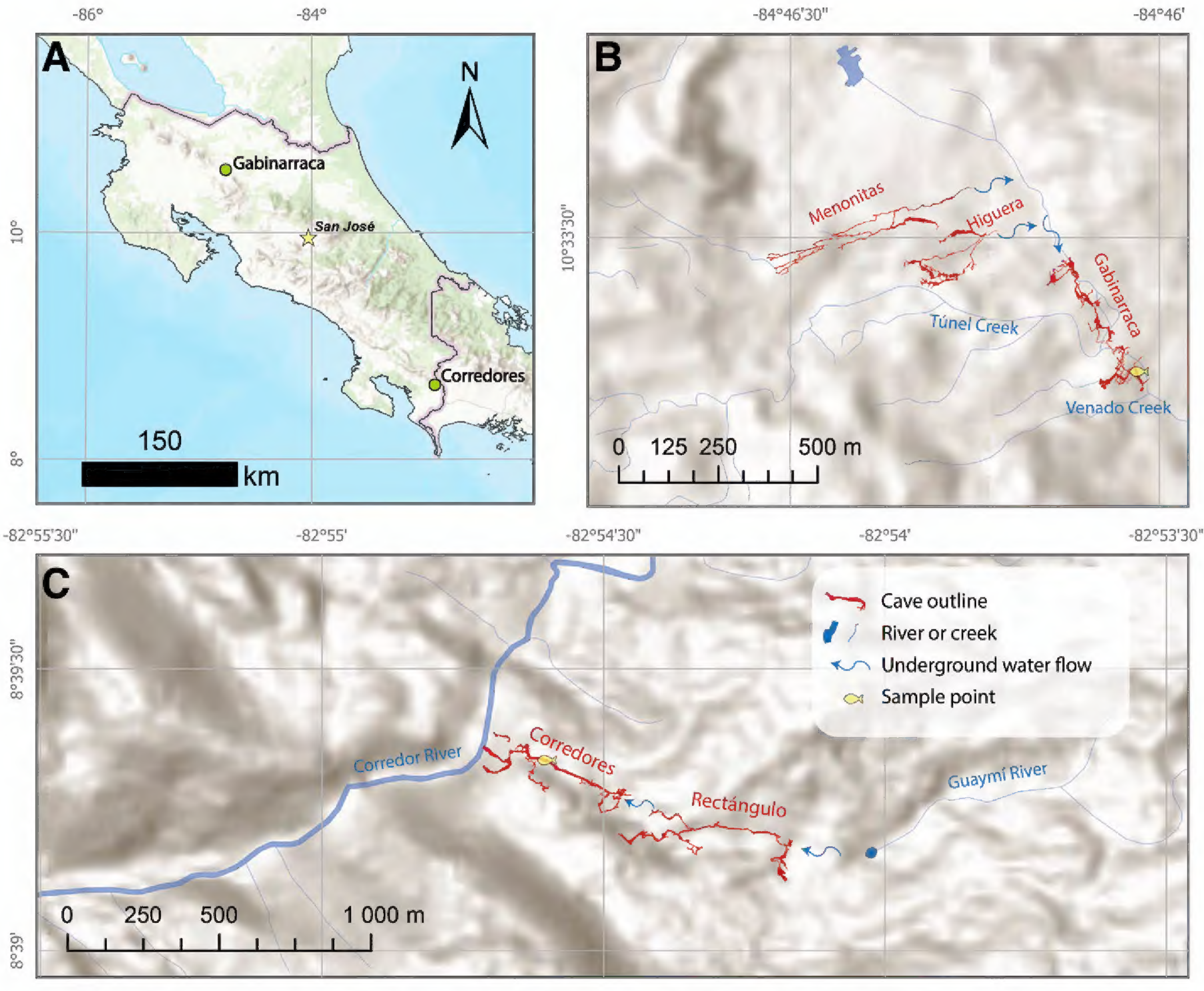

Figure 1. Location of the surveyed caves **A** map of Costa Rica displaying the location of Gabinarraca and Corredores caves (green dots) at the country-level scale (borders with Nicaragua to the north and Panama to the south highlighted in light purple). Maps displaying the location of the surveyed caves at the local scale, including geomorphological and hydrological features: **B** Gabinarraca and **C** Corredores.

Methods

Area of study

Gabinarraca and Corredores caves are located in the Costa Rican provinces of Alajuela and Puntarenas, respectively (Fig. 1A). Each cave constitutes the terminal segment of a karst system comprised of multiple interconnected caves with a perennial underground river (Fig. 1B, C). Gabinarraca cave is on the Caribbean versant of northwestern Costa Rica (although very near the continental water divide), c. 11 km northwest of the Arenal volcano. It is part of the Venado karstic system, which developed from Miocene limestone from the Venado Formation, and comprises three main caves: Menonitas (1620 m in length), Higuera (954 m in length), and Gabinarraca (2351 m in length), totaling almost 5 km of underground passages (Ulloa et al. 2021) (Fig. 1B). Not only is Gabinarraca cave the longest of the Venado karstic system caves, but it is also the best known, since it has been a tourist attraction for over two decades (Ulloa et al. 2021) (Fig. 2A, B). Corredores cave, on the other hand, is located on the Pacific versant of southern Costa Rica, very close to the border with Panama, c. 3.5 km east of Ciudad Neily (Fig. 1A). It is part of the Cerro Corredores karst system, which extends over a

Figure 2. Photographs displaying physical features of the surveyed caves and associated rivers/streams **A** Gabinarraca cave entrance, showing the outflow of water into Quebrada El Túnel **B** inside Gabinarraca cave while electrofishing **C** Quebrada El Túnel close to the entrance of Gabinarraca cave **D** Corredores cave entrance **E** Río Corredor as seen from the cave entrance higher up **F** Río Corredor riverbed at a small canyon on the way to the cave access point.

length of c. 3 km carrying water from the Guaymí River that infiltrates from the east through a sinkhole and flows underground northwest through several caves, including La Bruja (221 m), El Rectángulo (1411 m), and Corredores (1624 m), before emerging from the aquifer and flowing into the Corredor River (Peacock and Hempel 1993) (Figs 1C, 2D–F). The karst in the area develops in Eocene limestones associated with the Fila de Cal Formation (Chesnel and Rodríguez 2021).

Specimen sampling and preservation

Both sampled caves are wet (containing active watercourses but not fully flooded or submerged) and mostly horizontal, and therefore did not require specialized vertical caving techniques for surveying and sampling. Specimen sampling was accomplished using a combination of baited minnow traps and electrofishing. Inside the surveyed

caves, we collected fishes along the main longitudinal axis of the cave up to the point of maximum penetration, which was approximately a few hundred meters in both caves. In addition to the caves, we collected specimens of *Rhamdia* from epigean populations from streams and rivers mainly near Gabinarraca cave (Río Frío basin), including the very stream flowing out of the cave (Quebrada El Túnel) (Figs 2C, 3). Because of logistical issues, we were not able to secure samples of epigean *Rhamdia* from the Río Corredor, the river most closely associated to the Corredores cave. After capture, we euthanized the fishes using the anesthetic tricaine mesylate (MS-222) and then took tissue samples (fin clips) for the genetic component of our study. Tissues were preserved in 96% ethanol and eventually frozen at −20 °C. After tissuing, we fixed voucher specimens using a 10% formalin solution. Back in the lab, we washed formalin-fixed specimens and then gradually transferred them to 70% ethanol for long-term storage in the Colección Ictiológica del Museo de Zoología de la Universidad de Costa Rica (UCR), where they were catalogued and deposited (Table 1). Fishes were handled in accordance with recommended guidelines for the use of fishes in research (Jenkins et al. 2014). Specimens were collected under permit R-SINAC-SE-DT-PI-029-2023 issued by the Sistema Nacional de Áreas de Conservación (SINAC). Access permit to genetic resources (CBio-54-2022-#359) was extended by the Comisión Institucional de Biodiversidad of the UCR. Taxonomic identification of preserved specimens based on external morphology was conducted using relevant published keys and systematic revisions (Silfvergrip 1996; Bussing 1998; Hernández et al. 2015).

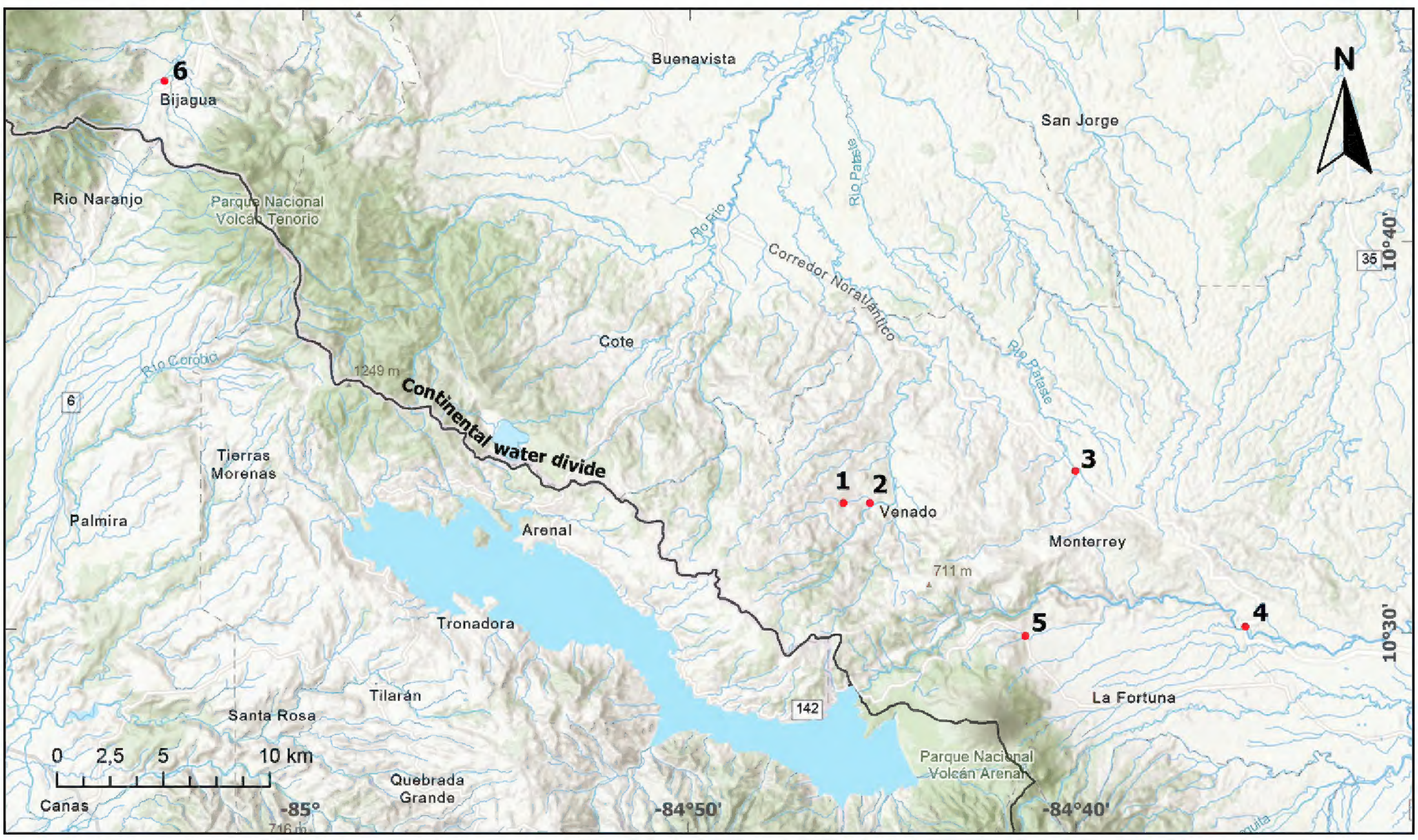

Figure 3. Collecting sites of epigean populations of *Rhamdia* sampled in this study **1** Quebrada El Túnel (at Gabinarraca cave entrance) **2** Quebrada El Túnel (further downstream from Gabinarraca cave entrance) **3** Río Pataste **4** Río Arenal **5** Quebrada Palma, and **6** Quebrada Altamira. All sites are within the Alajuela province, Atlantic versant. Black outline in map corresponds to the continental water divide.

Table 1. Sampling localities with their respective species, catalog/vouchers numbers, and GenBank accession numbers of sequences generated in this study and used in phylogenetic analyses. *at Gabinarraca cave entrance; **further downstream from Gabinarraca cave entrance.

Habitat	Locality	Coordinates	Municipality	Province	Basin	Versant	Species	Catalog (n)	Tissued vouchers	DNA voucher	GenBank Accession	
											COI	CYB
Hypogean	Gabinarraca cave	10°33'17"N, 84°46'01"W	Venado	Alajuela	Río Frío	Atlantic	*Rhamdia laticauda*	UCR 3323-01 (6)	JA1913-17	JA1913	PQ451515	PQ458956
										JA1914	PQ451516	PQ458957
										JA1915	PQ451517	PQ458958
										JA1916	PQ451492	PQ458959
							Rhamdia nicaraguensis	UCR 3323-02 (13)	JA1902-12	JA1902	PQ451502	PQ458966
										JA1903	n/a	PQ458967
										JA1904	PQ451503	PQ458968
										JA1905	PQ451504	PQ458969
										JA1906	PQ451505	PQ458970
										JA1907	PQ451506	PQ458971
										JA1908	PQ451507	PQ458972
										JA1909	PQ451508	PQ458973
										JA1910	PQ451509	PQ458974
										JA1911	PQ451497	PQ458975
										JA1912	PQ451510	PQ458976
	Corredores cave	08°39'23"N, 82°54'44"W	Ciudad Neily	Puntarenas	Río Coto	Pacific	*Rhamdia guatemalensis*	UCR 3330-01 (8)	JA2068-74	JA2068	PQ451499	PQ458950
										JA2070	n/a	PQ458951
										JA2071	PQ451500	PQ458952
										JA2072	PQ451501	PQ458953
										JA2073	n/a	PQ458954
										JA2074	n/a	n/a
Epigean	Quebrada El Túnel*	10°33'17"N, 84°46'01"W	Venado	Alajuela	Río Frío	Atlantic	*Rhamdia laticauda*	UCR 3324-01 (3)	JA1921-23	JA1921	PQ451518	PQ458960
										JA1922	PQ451493	PQ458961
										JA1923	PQ451519	PQ458962
							Rhamdia nicaraguensis	UCR 3324-02 (3)	JA1918-20	JA1918	PQ451511	PQ458977
										JA1919	PQ451512	PQ458978
										JA1920	PQ451513	PQ458979
	Quebrada El Túnel**	10°33'17"N, 84°45'20"W	Venado	Alajuela	Río Frío	Atlantic	*Rhamdia laticauda*	UCR 3325-01 (7)	JA1924-30	JA1927	PQ451520	n/a
										JA1928	PQ451494	PQ458963
							Rhamdia nicaraguensis	UCR 3325-02 (2)	JA1931-32	n/a	n/a	n/a

Habitat	Locality	Coordinates	Municipality	Province	Basin	Versant	Species	Catalog (n)	Tissued vouchers	DNA voucher	GenBank Accession	
											COI	CYB
Epigean	Río Arenal	10°30'09"N, 84°35'38"W	Venado	Alajuela	Río San Carlos	Atlantic	*Rhamdia laticauda*	UCR 3326-01 (8)	JA1952-59	JA1955	PQ451495	PQ458964
							Rhamdia nicaraguensis	UCR 3326-02 (2)	JA1960-61	n/a	n/a	n/a
	Río Pataste	10°34'07"N, 84°40'02"W	Venado	Alajuela	Río Frío	Atlantic	*Rhamdia laticauda*	UCR 3327-01 (4)	JA1943-46	JA1946	PQ451521	n/a
							Rhamdia nicaraguensis	UCR 3327-02 (7)	JA1936-42	JA1939	n/a	PQ458980
										JA1940	PQ451514	n/a
	Quebrada Altamira	10°44'01"N, 85°03'34"W	Bijagua	Alajuela	Río Zapote	Atlantic	*Rhamdia laticauda*	UCR 3328-01 (15)	JA1804-11	JA1804	PQ451496	PQ458955
	Quebrada Palma	10°29'54"N, 84°41'19"W	Palma	Alajuela	Río San Carlos	Atlantic	*Rhamdia nicaraguensis*	UCR 3329-01 (5)	JA1734-38	JA1734	PQ451498	PQ458965
							Rhamdia laticauda	UCR 3329-02 (5)	JA1729-33	n/a	n/a	n/a

Comparative data generation and analysis

To document patterns of phenotypic and genetic variation, and to shed light on the taxonomic nature of the cave-dwelling populations of *Rhamdia* reported herein, we generated meristic and morphometric data from hypogean samples as well as DNA sequence data from both hypogean and epigean samples. Morphometric measurements and meristic counts follow previous taxonomic studies of *Rhamdia* (Silfvergrip 1996; Hernández et al. 2015; Buenavad-González et al. 2023). All measurements were taken on the left side of specimens using a Mitutoyo digital caliper (precision = 0.1 mm; accuracy = ± 0.02 mm). Comparative genetic data consisted of partial fragments of the mitochondrial protein-coding genes cytochrome c oxidase subunit I (COI) and cytochrome b (CYTB). We generated COI and CYTB data from representatives of all cave-dwelling populations documented herein, as well as from samples of epigean *Rhamdia* collected during the fieldwork component of this study and relevant for comparative purposes (Table 1). To increase our precision in estimating the phylogenetic placement of the Costa Rican cave-dwelling populations with respect to the entire Middle American clade of the genus, we broadened the taxonomic and geographic coverage of samples used for phylogenetic analyses by including additional COI and CYTB sequences of *Rhamdia* retrieved from GenBank (www.ncbi.nlm.nih.gov/Genbank) and from unpublished data previously generated by the lead author (JA). To generate DNA sequence data, we first extracted total genomic DNA from fresh tissue samples using the Qiagen DNeasy Tissue Extraction Kit, following the manufacturer's protocol. We amplified and sequenced COI and CYTB following the procedures (primers and PCR thermal profiles) employed in recent molecular phylogenetic studies of the genus that used the same markers (Arroyave and De La Cruz Fernández 2021a, 2021b, Buenavad-González et al. 2023). DNA extraction, amplification, and sequencing were carried out at Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud (Instituto de Biología, UNAM) and Pritzker Molecular Laboratory at the Field Museum of Natural History (FMNH). Contig assemblage, sequence editing, multiple sequence alignment—using MUSCLE (Edgar, 2004)— and the calculation of uncorrected genetic distances (*p*-distances) were performed in Geneious Prime 2024.0.7 (https://www.geneious.com). The resulting COI and CYTB matrices consisted of 116 and 126 ingroup (trans-Andean/Middle American *Rhamdia*) terminals, including representatives from 9 and 10 of the 11 valid species in the clade, respectively. In addition to the individual gene alignments, we analyzed a concatenated alignment—assembled with the software 2matrix (Salinas and Little 2014)—taxonomically limited to 59 ingroup terminals (with voucher specimens) from nine species for which both COI and CYTB data were available. In all cases, the cis-Andean species *Rhamdia quelen* was used as the outgroup and root, based on previous phylogenetic research that supports the reciprocal monophyly between cis- and trans-Andean clades of *Rhamdia* (Perdices et al. 2002; Hernández et al. 2015; Arroyave and De La Cruz Fernández 2021b). For each alignment, statistical selection of the best-fit model of nucleotide substitution was implemented with the software jModelTest2 (v. 2.1.10) (Darriba et al. 2012) under the following likelihood settings:

number of substitution schemes = 3; base frequencies = +F; rate variation = +I and +G with nCat = 4; base tree for likelihood calculations = ML optimized; and base tree search = NNI, effectively evaluating 24 alternative models. We inferred a phylogenetic tree for each individual gene matrix as well as for the concatenated alignment using the software RAxML-NG (v. 1.2.1) (Kozlov et al. 2019) under their respective best-fit models of molecular evolution. Clade support was estimated using the bootstrap character resampling method (Felsenstein 1981) based on 1000 pseudoreplicates.

Results

Our sampling efforts in the surveyed caves (Corredores and Gabinarraca) resulted in the discovery of three cave-dwelling populations of *Rhamdia*, identified as members of the species *R. guatemalensis* (Corredores cave; n = 8) (Figs 4, 7), *R. laticauda* (Gabinarraca cave; n = 6) (Figs 5, 7), and *R. nicaraguensis* (Gabinarraca cave; n = 13) (Figs 6, 7). Furthermore, we collected individuals from epigean populations of both *R. laticauda* and *R. nicaraguensis*, from six and five localities, respectively (Table 1). Taxonomic identifications were initially based on external morphology and further corroborated by comparative molecular data in a phylogenetic context (Figs 8–10). All hypogean populations displayed partial loss of body pigmentation, resulting in a yellowish coloration in life, which contrasts with the dark, melanic phenotype typical of epigean forms (Fig. 6D). Notably, none of the hypogean populations exhibited a pattern of eye reduction/loss, except for a single specimen of *R. guatemalensis* from the Corredores cave which displayed a slight and asymmetric eye degeneration and reduction (Fig. 4A).

Morphological and meristic data from the specimens collected at the surveyed caves are presented in Tables 2, 3. Inter- and intraspecific variation in morphological traits of taxonomic importance (i.e., those used to distinguish among the three species of *Rhamdia* present in Costa Rica) such as pectoral spine serration, interdorsal space, length of barbels, and head length, conformed with the expectations of our species designations.

GenBank accession numbers corresponding to the DNA sequence data generated in this study (COI and CYTB) are presented in Table 1. Regardless of marker/matrix, the inferred phylogenies (Figs 8–10) unambiguously place the Costa Rican cave-dwelling populations of *Rhamdia* documented herein (colored in red) well nested within the *R. guatemalensis* clade (in the case of samples from the Corredores cave) and the "*R. laticauda*-group" clade (Weber and Wilkens 1998) (in the case of samples from the Gabinarraca cave). Although these phylogenies consistently recover *R. laticauda* deeply paraphyletic, samples from Gabinarraca morphologically identified as *R. laticauda* were resolved within a well-supported clade consisting exclusively of Costa Rican samples of this species, both hypogean and epigean (Figs 8–10). Similarly, samples from Gabinarraca cave identified morphologically as *R. nicaraguensis* nested within a moderately to well-supported clade consisting exclusively of *R. nicaraguensis* samples, including epigean ones. Notably, within the "*R. laticauda*-group" clade, *R. nicaraguensis* was consistently recovered as monophyletic (Figs 8–10).

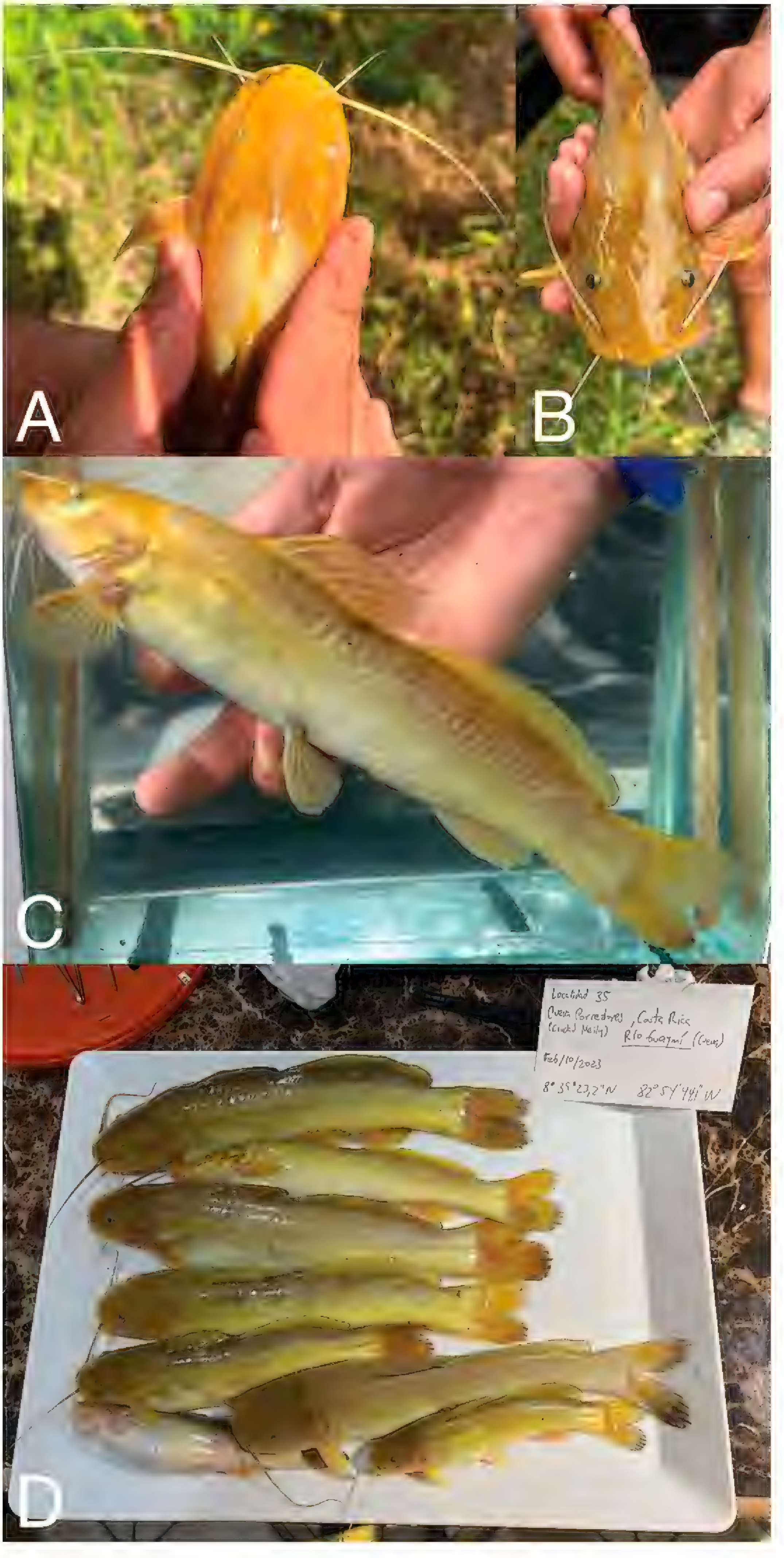

Figure 4. Photographs of hypogean *Rhamdia guatemalensis* from Corredores cave **A, B** dorsal views of head and anterior part of body in life after capture **C** lateral view of live specimen in aquarium **D** totality of specimens collected (n = 8), immediately postmortem and before tissuing and preservation.

Table 2. Meristic comparative data from samples of hypogean populations of *Rhamdia* spp. from Gabinarraca and Corredores caves. Meristic traits abbreviations as follows: PFR = pectoral-fin rays, PvFR = pelvic-fin rays, DFR = dorsal-fin rays, ARF = anal-fin rays, uCFR = upper caudal-fin rays, and lCFR = lower caudal-fin rays. Caudal-fin rays numbers (x,y,z) correspond to unsegmented (x), unbranched segmented (y), and branched segmented (z) rays.

Cave	Species	Catalog	Voucher	PFR	PvFR	DFR	AFR	uCFR	lCFR
Gabinarraca	*Rhamdia laticauda*	UCR 3323-01	3323-01-A	I-9	6	I-6	9	3,2,8	3,2,9
			JA1906	I-9	7	I-6	9	3,2,9	3,2,9
			JA1902	I-9	7	I-5	10	3,2,9	3,2,8
			3323-01-B	I-9	7	I-6	9	3,2,8	3,2,10
	Rhamdia nicaraguensis	UCR 3323-02	JA1903	I-9	7	I-5	9	3,2,6	3,2,8
			JA1904	I-9	7	I-6	10	3,2,9	3,2,10
			JA1905	I-9	7	I-6	9	3,2,8	3,2,9
			JA1907	I-9	7	I-6	9	3,2,9	3,2,9
			JA1908	I-9	8	I-6	9	3,2,8	3,2,8
			JA1909	I-9	7	I-6	9	3,2,9	3,2,9
			JA1910	I-9	7	I-6	9	3,2,9	3,2,9
			JA1911	I-9	7	I-6	9	3,2,7	3,2,9
			JA1912	I-9	7	I-6	9	3,2,9	3,2,8
Corredores	*Rhamdia guatemalensis*	UCR 3330-01	3330-01-A	I-7	6	I-6	9	3,2,9	3,2,10
			JA2068	I-8	7	I-6	9	3,2,9	3,2,10
			JA2069	I-9	7	I-6	9	3,2,9	3,2,10
			JA2070	I-9	6	I-6	8	3,2,8	3,2,9
			JA2071	I-8	7	I-6	8	3,2,9	3,2,9
			JA2072	I-7	6	I-7	8	3,2,9	3,2,7
			JA2073	I-9	7	I-6	9	3,2,9	3,2,9
			JA2074	I-9	7	I-6	9	3,2,8	3,2,9

Discussion

In the most recent and comprehensive review of Costa Rican cave-dwelling fauna, Deleva et al. (2023) reported on the presence of hypogean populations of *R. guatemalensis* in the Corredores and Bananal cave systems, as well as in other adjacent caves. Our results confirm the presence of *Rhamdia* catfishes in the Corredores cave (Figs 4, 7–10). The existence of hypogean populations of *R. guatemalensis* in other caves from southern Costa Rica—such as Bananal—has yet to be verified; however, these may seem likely given our Corredores cave findings and previous reports from the region (anecdotal and otherwise). The existence of cave-dwelling populations of *R. guatemalensis* in Costa Rica is noteworthy considering that most hypogean populations of *Rhamdia* that have been documented so far are derivatives of *R. laticauda*, either as cave-adapted populations or as species-level lineages within the "*R. laticauda*-group" clade (Arroyave and De La Cruz Fernández 2021a, 2021b, Buenavad-González et al. 2023). To our knowledge, the Corredores cave population effectively constitutes the fourth taxonomically verified record of a hypogean population of *R. guatemalensis*; the first three being from Mexican caves in the states of Tabasco (Grutas de Coconá) and Chiapas (Los Bordos and El Encanto caves) (Buenavad-González et al. 2023).

Figure 5. Photograph of hypogean *Rhamdia laticauda* from Gabinarraca cave. Totality of specimens collected (n = 6), immediately postmortem and before tissuing and preservation.

Despite the Corredores cave population not exhibiting full troglomorphism (due to the presence of eyes), it appears to be the most depigmented of all four *R. guatemalensis* hypogean populations documented to date (Fig. 4).

Remarkably, among our findings is the discovery of syntopic hypogean populations from two different *Rhamdia* species (*R. laticauda* and *R. nicaraguensis*) inhabiting the Gabinarraca cave system. This finding is noteworthy because it represents the first taxonomically verified record of a cave-dwelling population of *R. nicaraguensis*. Furthermore, the population of *R. laticauda* from Gabinarraca cave constitutes the latest addition to the extensive list of hypogean records for this epigean species—which includes five populations from the karstic Sierra de Zongolica in the state of Veracruz (Arroyave and De La Cruz Fernández 2021a) and two populations from karstic caves

Table 3. Morphometric comparative data from samples of hypogean populations of *Rhamdia* spp. from Gabinarraca and Corredores caves. Measurements abbreviations as follows: SL = Standard Length, HL = Head Length, HL = Head Length, BW = Body Width, DFH = Dorsal Fin Height, DSH = Dorsal-fin Spine Height, AFL = Anal Fin Length, AdFL = Adipose Fin Length, PFL = Pectoral Fin Length, PSL = Pectoral-fin Spine Length, PvFL = Pelvic Fin Length, ISL = Interdorsal Space Length, CPL = Caudal Peduncle Length, CPD = Caudal Peduncle Depth, IOW = Interorbital Width, ORB = Orbital Diameter, SNT = Snout Length, MBL = Maxillary Barbel Length, MdBL = Mandibular Barbel Length, MeBL = Mental Barbel Length.

	Gabinarraca Cave						**Corredores Cave**		
	***Rhamdia laticauda* (n = 4)**			***Rhamdia nicaraguensis* (n = 9)**			***Rhamdia guatemalensis* (n = 8)**		
	Range	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**
				mm					
SL	119.94–198.00	150.45	34.85	107.16–171.00	132.82	21.23	124.41–237.00	193.43	45.70
HL	29.75–43.29	34.45	6.04	24.80–40.43	31.32	5.50	28.60–52.92	43.10	10.27
				% SL					
HL	21.46–24.99	23.14	1.74	22.64–24.64	23.53	0.67	20.49–22.99	22.30	0.78
BW	16.35–17.92	17.19	0.64	15.48–18.14	16.56	0.76	18.00–18.91	18.48	0.32
DFH	13.25–14.75	14.00	0.64	10.17–14.88	13.34	1.28	12.48–15.64	13.93	1.03
DSH	5.32–6.20	5.81	0.37	4.19–5.05	4.55	0.29	5.98–6.72	6.35	0.28
AFL	17.29–18.80	17.95	0.63	18.32–21.42	20.15	1.05	17.61–19.60	18.59	0.60
AdFL	40.80–42.41	41.93	0.75	39.90–45.58	43.84	1.99	36.54–38.39	37.74	0.57
PFL	10.49–13.89	12.22	1.50	12.45–13.79	13.15	0.45	12.16–13.89	12.95	0.57
PSL	6.67–8.58	7.45	0.89	7.02–8.89	7.88	0.62	7.30–9.24	8.41	0.62
PvFL	9.90–12.61	11.32	1.40	11.43–12.94	12.08	0.47	11.01–13.12	11.63	0.83
ISL	4.61–4.78	4.69	0.08	1.46–2.43	1.67	0.30	5.27–7.75	6.67	0.85
CPL	16.35–18.94	17.47	1.22	17.79–18.96	18.24	0.41	17.90–20.23	18.48	0.75
CPD	9.95–10.35	10.11	0.17	9.78–11.12	10.30	0.40	9.71–11.36	10.83	0.55
				% HL					
IOW	36.13–42.67	38.94	2.72	35.02–39.45	37.13	1.46	40.05–46.97	43.71	2.14
ORB	17.39–20.10	18.65	1.23	15.94–20.24	17.98	1.44	9.01–16.09	12.07	2.16
SNT	37.75–41.49	39.48	1.60	35.54–41.75	38.15	1.77	32.72–41.85	36.82	2.73
MBL	100.11–117.27	111.31	7.64	88.97–136.11	121.53	14.67	124.49–228.91	158.01	33.37
MdBL	47.62–54.66	50.70	3.01	54.28–74.97	60.26	5.95	62.72–90.31	72.73	9.92
MeBL	27.66–36.79	32.88	3.97	32.69–46.50	35.71	4.21	41.76–55.44	47.45	5.06

in the state of Chiapas (Buenavad-González et al. 2023)—further supporting the notion that *R. laticauda* is the quintessential cave colonizer species in the genus. Lastly, and perhaps more interestingly, this is the first time that cave-dwelling populations from different species of *Rhamdia* are reported to be living in syntopy. Coexistence of multiple species of hypogean fishes in the same cave is not widespread but neither uncommon; around 40% of obligate cavefish species co-occur with other such species, although very rarely totaling more than two syntopic species per cave (Trajano 2001). Approximately half of the known instances of syntopy in cavefishes involve species relatively distant phylogenetically, while the other half involve species from the same family although mostly from different genera (Niemiller and Soares 2015). Apart from the case of *Rhamdia* in the Gabinarraca cave reported herein, the only other known instances of intrageneric syntopy in cavefishes are restricted to viviparous brotulas of the genus *Lucifuga* Poey 1858 in two Cuban caves, with three and two syntopic species,

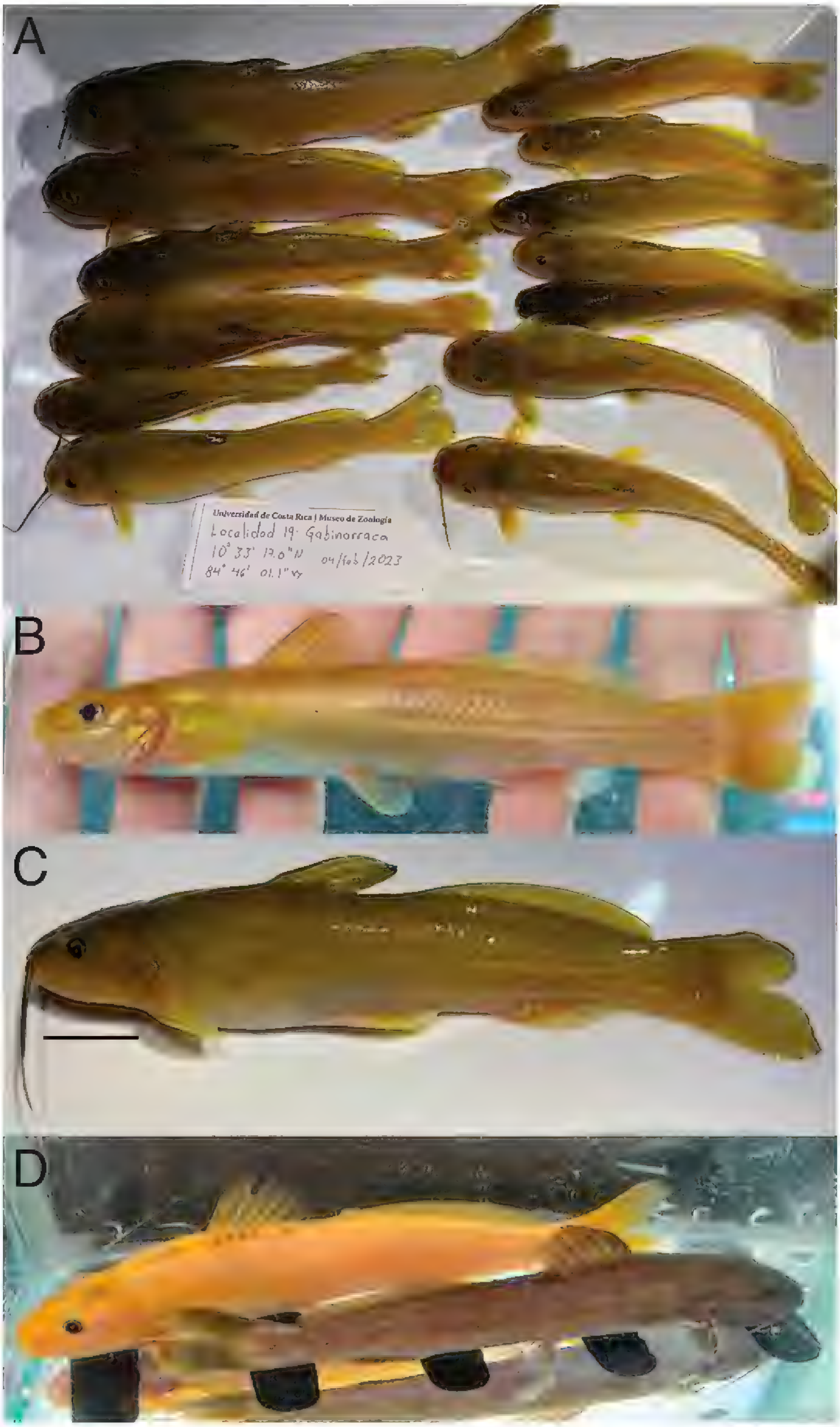

Figure 6. Photographs of hypogean *Rhamdia nicaraguensis* from Gabinarraca cave **A** totality of specimens collected (n = 13), immediately postmortem and before tissuing and preservation **B** lateral view of live specimen in aquarium **C** lateral view of specimen immediately postmortem. Metacercariae (two) of the parasitic fluke *Clinostomum* sp. are visible at the base of the anal fin **D** live specimens (in aquarium) of hypogean (Gabinarraca, yellow) and epigean (Quebrada El Túnel, dark brown) *R. nicaraguensis*. Scale bar: 1 cm.

respectively (Trajano 2001). It is generally assumed that coexistence in syntopy requires some type of resource (mainly food and space) partitioning so as to minimize competition (Pianka 1973), and this is especially true of closely related and morphologically

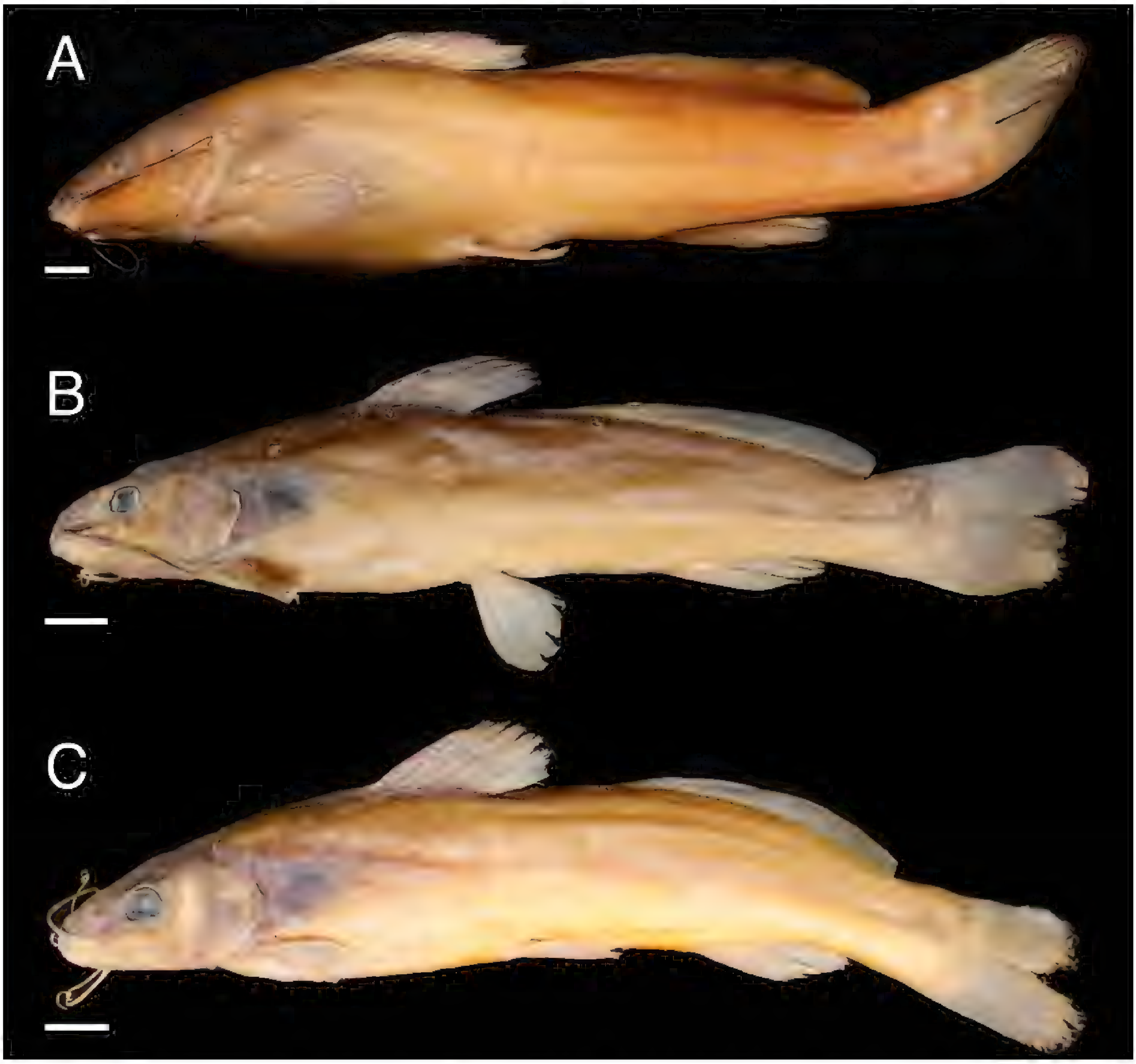

Figure 7. Photographs of preserved representatives of the populations of cave-dwelling *Rhamdia* from Costa Rica reported in this study **A** *R. guatemalensis* from Corredores cave (UCR 3330-01; 230 mm TL) **B** *R. nicaraguensis* from Gabinarraca cave (UCR 3323-02; 165 mm TL), and **C** *R. laticauda* from Gabinarraca cave (UCR 3323-01; 158 mm TL). Scale bar: 1 cm.

similar species co-occurring in hypogean habitats that are generally oligotrophic and with simplified food webs (Niemiller and Soares 2015). In some well-documented cases, habitat partitioning and/or differences in diet between syntopic cavefish species have been posited as the evolutionary and ecological mechanisms allowing coexistence with minimum competition (Trajano 2001). This pattern of ecological differentiation and resource partitioning in cavefishes, however, has only been demonstrated in some instances of syntopy involving relatively phylogenetically distant species (from different genera and families). In cases of closely related and morphologically conserved syntopic cavefish species such as *Lucifuga* spp. from Cuban caves, the mechanisms enabling local coexistence are not fully understood, although it appears that competitive-driven shifts in diet and habitat preferences have yet to evolve (Trajano 2001). With hypogean populations of two species of *Rhamdia* (*R. laticauda* and *R. nicaraguensis*)

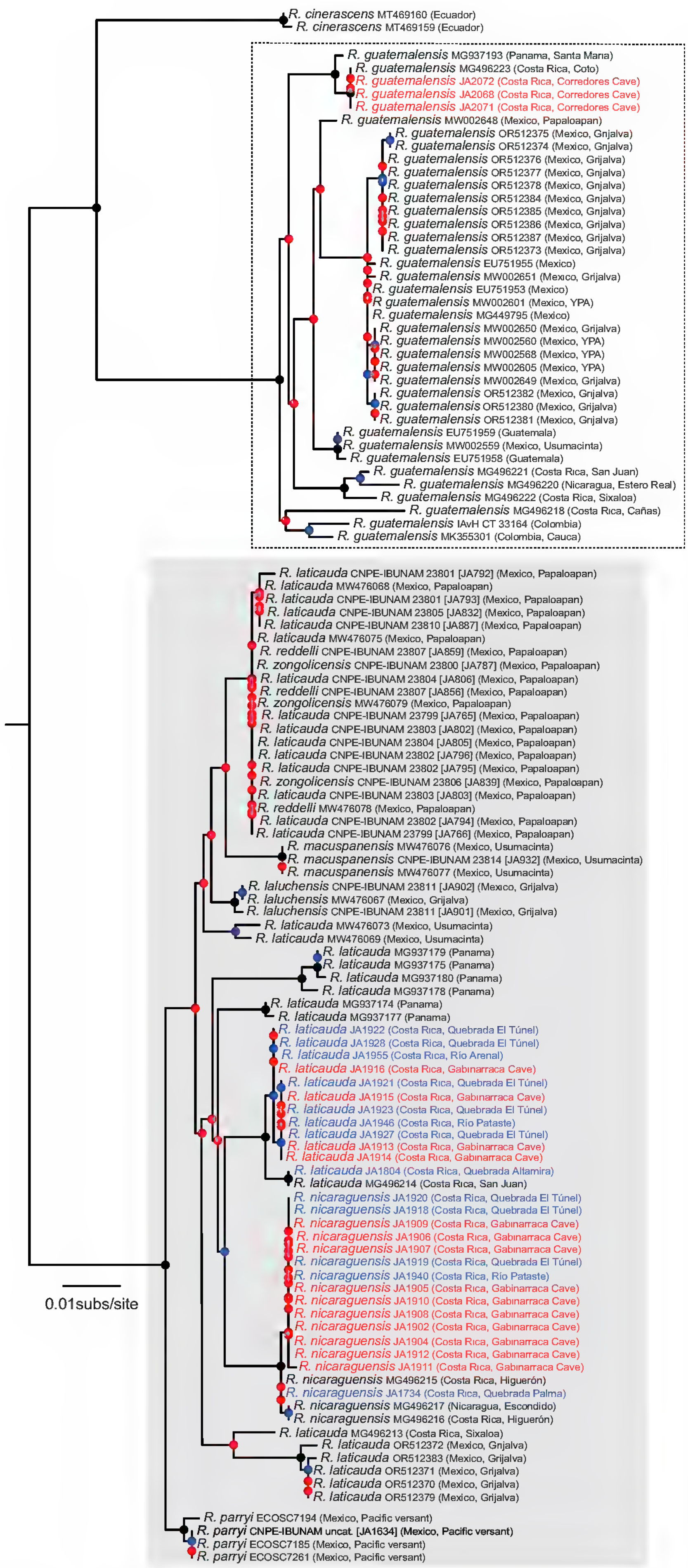

Figure 8. Phylogenetic relationships of Middle American *Rhamdia* inferred from comparative COI data. Colored circles on nodes indicate degree of clade support as determined by bootstrap values: black > 95, 95 ≥ blue ≥ 75, red < 75. Terminal names as follow: Species name + GenBank accession or catalog/voucher (for new/unpublished sequences) + country, basin (in parenthesis). Terminals corresponding to samples from populations of Costa Rican hypogean *Rhamdia* (documented herein) in red. Terminals corresponding to samples from Costa Rican epigean *Rhamdia* collected in this study in blue. Outgroup taxon (*Rhamdia quelen*) not shown. The dashed rectangle indicates the *R. guatemalensis* clade, whereas the light gray rectangle indicates the "*R. laticauda*-group" clade, inclusive of *R. nicaraguensis*, *R. parryi*, and the four Mexican stygobitic species.

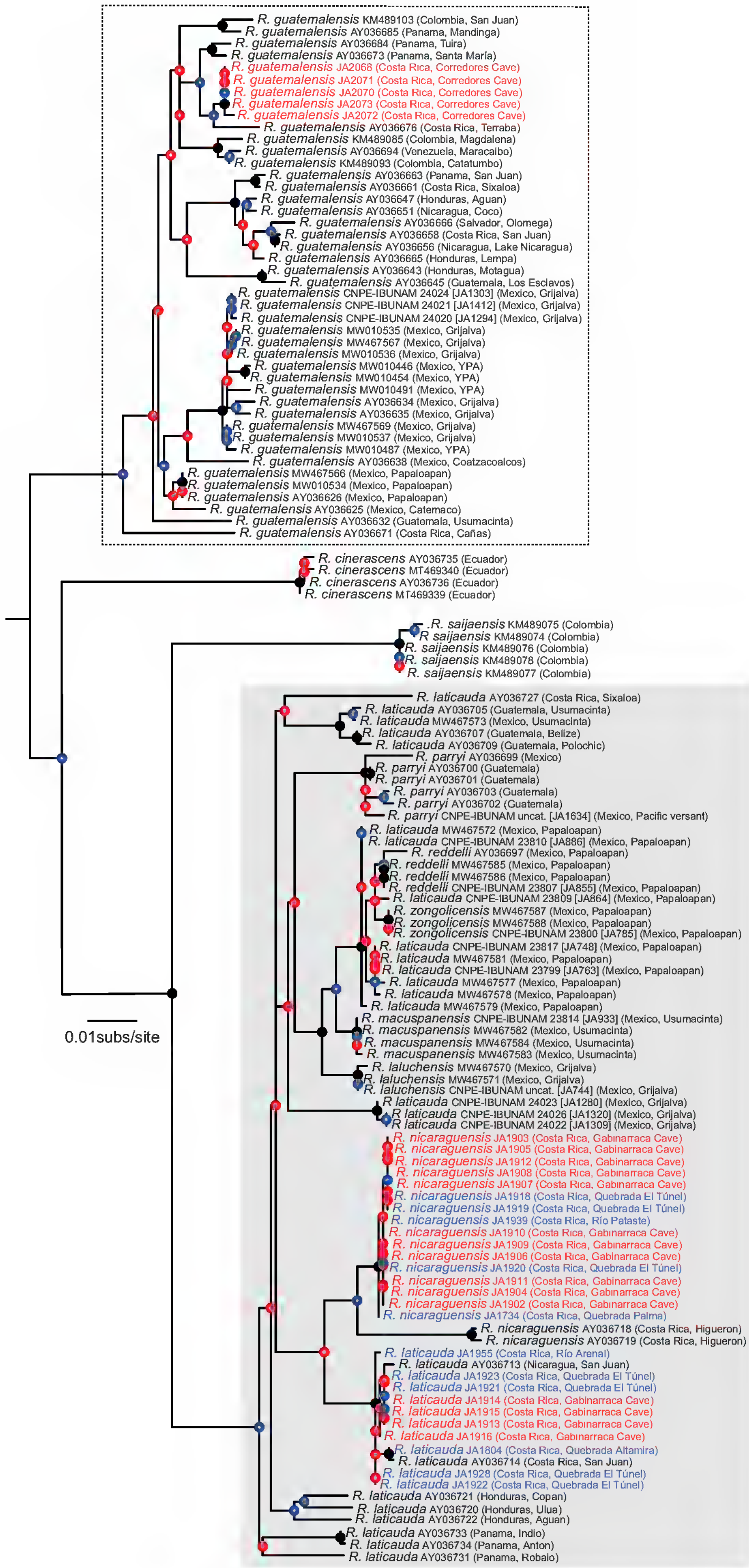

Figure 9. Phylogenetic relationships of Middle American *Rhamdia* inferred from comparative CYTB data. Colored circles on nodes indicate degree of clade support as determined by bootstrap values: black > 95, 95 ≥ blue ≥ 75, red < 75. Terminal names as follow: Species name + GenBank accession or catalog/voucher (for new/unpublished sequences) + country, basin (in parenthesis). Terminals corresponding to samples from populations of Costa Rican hypogean *Rhamdia* (documented herein) in red. Terminals corresponding to samples from Costa Rican epigean *Rhamdia* collected in this study in blue. Outgroup taxon (*Rhamdia quelen*) not shown. The dashed rectangle indicates the *R. guatemalensis* clade, whereas the light gray rectangle indicates the "*R. laticauda*-group" clade, inclusive of *R. nicaraguensis*, *R. parryi*, and the four Mexican stygobitic species.

living in syntopy, Gabinarraca cave in Costa Rica effectively constitutes a new model to investigate the processes allowing the local coexistence of closely related, morphologically similar, and seemingly ecologically equivalent species/populations of cavefishes. Future research aimed at generating and analyzing detailed data on diet and microhabitat occupation will be necessary to start shedding light on the ecological mechanisms allowing syntopy of *Rhamdia* catfishes in this cave system.

Given the ample taxonomic and geographic coverage, the resulting phylogenies (Figs 8–10) offer an extensive panorama of interspecific relationships within the Middle American *Rhamdia* clade and of phylogeographic structure within wide-ranging species such as *R. guatemalensis* and *R. laticauda*. As such, our phylogenetic results corroborate the relentless paraphyly of *R. laticauda* (with respect to *R. nicaraguensis*, *R. parryi*, and the Mexican stygobitic species) (Perdices et al. 2002; Arroyave and De La Cruz Fernández 2021b) and support the taxonomic distinctiveness (from *R. laticauda*) and monophyletic status of both *R. nicaraguensis* (Silfvergrip 1996; Bussing 1998) and *R. parryi* (Miller 2005), although uncertainty regarding their phylogenetic placement within the larger "*R. laticauda*-group" clade remains (Perdices et al. 2002; Arroyave and De La Cruz Fernández 2021b; Buenavad-González et al. 2023).

Phylogeographic structure in *R. guatemalensis* and *R. laticauda* appears generally consistent with a latitudinal gradient and the expectations from catchment hydrology (Perdices et al. 2002; Hernández et al. 2015; Arroyave et al. 2021). However, within these species-level clades, most internodes are short and poorly supported, rendering their phylogeographic histories unclear. The morphology-based species designations of the cave-dwelling *Rhamdia* populations reported herein were in all cases corroborated with molecular data in a phylogenetic framework (Figs 8–10). As expected from a biogeographic perspective, besides being resolved within their respective species-level clade, these hypogean populations always associated most closely with the geographically/hydrologically closest epigean populations. Samples from the Corredores cave (near the border with Panama) are more closely related to epigean populations of *R. guatemalensis* from Costa Rica and Panamá. Likewise, hypogean *Rhamdia* from Gabinarraca cave (*R. laticauda* and *R. nicaraguensis*) are more closely related—and practically genetically identical (i.e., p-distances < 0.3%)—to epigean samples of their respective species from the same basin (Río Frío) (Figs 8–10).

This pattern, also documented for numerous cave-dwelling populations of *Rhamdia* catfishes in southern Mexico (Arroyave and De La Cruz Fernández 2021a, 2021b, Buenavad-González et al. 2023), coupled with the observed relatively incipient troglomorphism (partial depigmentation but without eye loss), suggests recent cave colonization events and/or continued gene flow between epigean and hypogean populations. Further research into this subject, ideally based on genome-wide comparative data, would be required to properly test hypoteses of gene flow and the timing of lineage divergences and cave colonizations in *Rhamdia*. Similarly, further research is needed to shed light on basic yet poorly known aspects about the ecology of cave *Rhamdia*, such as diet and demography. Although we currently lack data about their trophic ecology and population dynamics, we assume that these hypogean populations are resident, and hypothesize that their incipient troglomorphism has evolved as a result of living

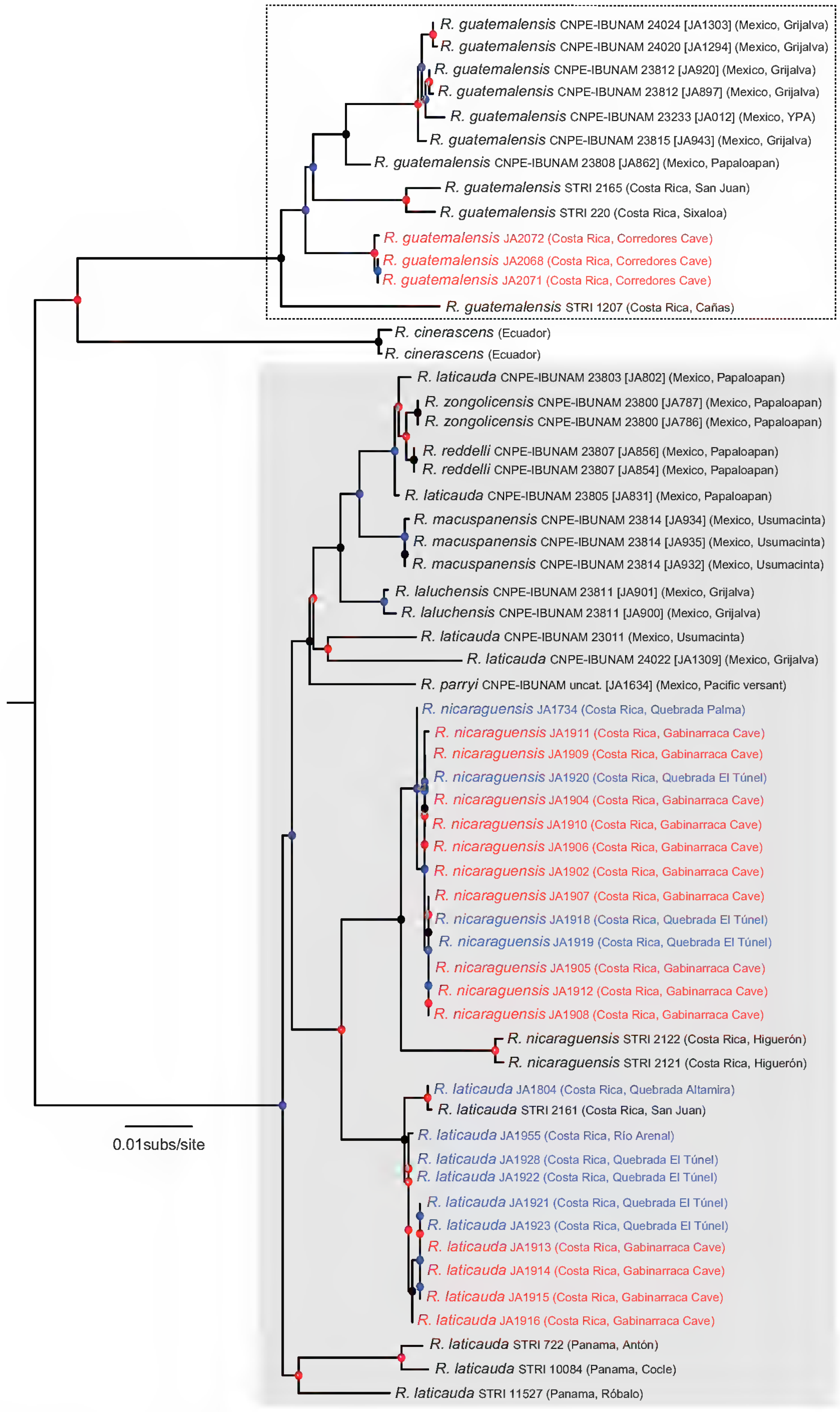

Figure 10. Phylogenetic relationships of Middle American *Rhamdia* inferred from comparative COI + CYTB data (concatenated). Colored circles on nodes indicate degree of clade support as determined by bootstrap values: black > 95, 95 ≥ blue ≥ 75, red < 75. Terminal names as follow: Species name + GenBank accession or catalog/voucher (for new/unpublished sequences) + country, basin (in parenthesis). Terminals corresponding to samples from populations of Costa Rican hypogean *Rhamdia* (documented herein) in red. Terminals corresponding to samples from Costa Rican epigean *Rhamdia* collected in this study in blue. Outgroup taxon (*Rhamdia quelen*) not shown. The dashed rectangle indicates the *R. guatemalensis* clade, whereas the light gray rectangle indicates the "*R. laticauda*-group" clade, inclusive of *R. nicaraguensis*, *R. parryi*, and the four Mexican stygobitic species.

in subterranean environments, a hypothesis subject to future testing. The results from recent population genomics studies conducted on other cavefish complexes such as *Astyanax mexicanus* (Garduño-Sánchez et al. 2023) and *Typhlichthys subterraneus* (Hart et al. 2024) suggest that analyses of genome-wide data in a phylogeographic framework are a promising avenue for investigating modes of cave-adapted evolution in other fishes with cave-dwelling populations such as *Rhamdia*.

In conclusion, this study adds to a body of work showcasing the continued discovery of cave-dwelling populations of *Rhamdia* catfishes during targeted ichthyological surveys in karstic regions of Middle America (Arroyave and De La Cruz Fernández 2021a, Buenavad-González et al. 2023), and as such, it reinforces the notion that our understanding of the diversity of hypogean *Rhamdia* is only partial and that sustained exploration and taxonomically sound documentation work are paramount to advancing knowledge about the diversity and evolution of these group of Neotropical catfishes. We hope that by uncovering and taxonomically documenting cavefish diversity present in Costa Rica and its karstic systems, this study opens the gates for future research on the subject in the region.

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